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MAINTENANCE OF GENOME INTEGRITY VIA ACTIVITIES OF THE COHESIN NETWORK

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ABSTRACT

Correct segregation of sister chromatids is an important mechanism for keeping the genome intact. The cohesin complex holds the sister chromatids together from the time of their formation during replication, until separation at anaphase and is thereby mediating cohesion between the sister chromatids, essential for correct chromatid segregation. Members of the cohesin network in addition play essential roles in the repair of double strand breaks (DSBs), and have been shown to be involved in regulation of transcription. Thus, cohesin is a master regulator of a majority of the cellular processes required for transfer of the correct genetic information from one cell generation to the next. The aim with this thesis was to further elucidate the function(s) of the cohesin network in genome integrity. In doing so, either budding yeast or human cell cultures were used. In budding yeast, cohesin is recruited to the vicinity of an induced DSB and cohesion is established genome wide. This phenomenon of re-establishment of cohesion is called Damage induced (DI-) cohesion. By investigating the function of Polymerase η in DI-cohesion, we found that it is differentially regulated at the break site and genome-wide. We also suggested that the function of break proximal DI-cohesion is to support DSB repair, while the genome wide DI-cohesion is important for correct chromosomal segregation and for survival following repeated break induction. A gene regulatory role of cohesin and its loading complex Scc2/4 has been described in several organisms, but not investigated thoroughly in yeast. Thus, we investigated the gene transcription profiles in Scc2-deficient cells in the absence and presence of DNA damage. We conclude that Scc2 is indeed instrumental for gene regulation also in budding yeast, both globally in an undamaged situation, and in response to DSB induction. Our data also indicate that the difference in gene response between WT and Scc2-deficient cells is not based on overt changes in cohesin binding. Mutations in *NIPBL* (human ortholog of *SCC2*), are frequent in Cornelia de Lange syndrome (CdLS) patients. By studying the DSB repair in B-cells originated from CdLS patient, we found a shift towards the alternative, microhomology-based, end joining pathway during class switch recombination, implicating that *NIPBL* is important for classical NonHomologous End Joining (NHEJ). Our results suggest that *NIPBL* plays an important and conserved role for NHEJ, in addition to its previously known function in homologous recombination. Altogether I have with this thesis highlighted the importance of the cohesin network in DI-cohesion and DNA DSB repair, as well as in the transcriptional regulation, all important components of the systems used for maintenance of genome integrity.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Att celledelning är elementärt under fosterutvecklingen och uppväxten är lätt att förstå. Men celledelning fortgår hela livet, dels för att ersätta celler som gått förlorade t ex vid skador och sjukdomstillstånd och dels under den normala förnyelsen av våra organ och vävnader. Vid varje celledelning måste arvsmassan (DNA), i form av kromosomer, kopieras för att sedan fördelas i två exakt likadana uppsättningar till varje ny cell. Detta eftersom DNA innehåller koden för alla cellens funktioner och att misstag i denna process kan få allvarliga konsekvenser så som cancer om det är våra vanliga celler som drabbats, men också missfall eller barn med kromosomavvikelser, om misstaget sker i ett ägg eller en spermie. De nybildade kromosomkopiorna hålls samman av ett proteinkomplex som kallas cohesin, vars funktion kan beskrivas som ett kromosomlim. Detta är viktigt för att separationen av kopiorna vid celledelningen ska bli rätt, men också för reparation av DNA-skador, eftersom en oskadad nära kopia kan användas som mall för en skadad. Dessa processer styrs indirekt av ytterligare ett proteinkomplex (Scc2/4), eftersom det laddar Cohesin på DNA. Varje dag utsätts våra celler för olika typer av stress som leder till skador på DNA. Därför ses DNA reparation som en av de viktigaste processerna i en cell. Cohesin och därmed också Scc2 är båda involverade i DNA reparationen. För att förstå mer om detta studerar vi i den första studien hur och varför cohesin håller ihop kromosomkopiorna "extra mycket" när DNA skadas. Detta gör vi i jästceller som är en utmärkt modell eftersom de flesta DNA reparations proteiners utseende och funktioner är bibehållna under evolutionen. Om fel uppstår i Scc2 proteinet, resulterar det i ett medfött syndrom som kallas Cornelia de Lange syndromet (CdLS). CdLS är lyckligtvis ett sällsynt tillstånd men medför allvarliga mentala och fysiska utvecklingsskador. Detta tyder på att Scc2 har ytterligare funktioner utöver den för celledelning. Man har t ex sett ökad känslighet mot DNA skador och felaktig genreglering, vilket är det som bestämmer hur mycket av ett visst protein som ska finnas i en cell vid varje givet tillfälle. Att uttrycket av våra gener styrs korrekt är extra viktigt under fosterstadiet då celledelningen är snabb och alla vävnader och organ anläggs. Allt detta tillsammans understryker vikten av ett fungerande Scc2/4 komplex. I den andra studien undersökte vi hur vida avsaknad av Scc2 påverkar genreglering i jästceller. Om så vore fallet skulle mekanismen för genregleringsfunktionen kunna studeras mer renodlat, i ett system där vi tillfälligt helt kan stänga av proteinerna i fråga. I den tredje studien undersökte vi om brist på funktionellt Scc2, påverkar cellernas känslighet mot DNA skador samt möjligheten att reparera DNA dubbelstrandsbrott.

LIST OF PUBLICATIONS

This thesis is based on the following articles and manuscripts. They will be referred to in the text by their roman numerals.

- I. Enervald, E., * **Lindgren, E.**, * Katou, Y., Shirahige, K., Ström, L.
Importance of Pol η for Damage-Induced Cohesion Reveals Differential Regulation of Cohesion Establishment at the Break Site and Genome-Wide
PLoS Genet. (2013) 9:e1003158
* Equal contribution
- II. **Lindgren, E.**, Hägg, S., Giordano, F., Björkegren, J., Ström, L.
Inactivation of the Cohesin Loader Scc2 alters Gene Expression Both Globally and in Response to a Singel DNA Double Strand Break
Manuscript
- III. Enervald, E., Du, L., Visnes, T., Björkman, A., **Lindgren, E.**, Wincent, J., Borck, J., Colleaux, L., Cormier-Daire, V., C. van Gent, D., Pie, J., Puisac, B., de Miranda, N., Kracker, S., Hammarström, L., Villartay, J.P., Durandy, A., Schoumans, J., Ström, L., Pan-Hammarström, Q.
A regulatory role for the cohesin loader NIPBL in nonhomologous end joining during immunoglobulin class switch recombination
J. Exp. Med (2013) 210:2503-13

Publications not included in this thesis:

Huttner, H. B., Bergmann, O., Salehpour, M., Rácz, A., **Lindgren, E.**, Csonka, T., Csiba, L., Hortobágyi, T., Méhes, G., Englund, E., Solnestam, B., W., Zdunek, S., Scharenberg, C., Ström, L., Ståhl, P., Lundeberg, J., Dahl, A., Lindvall, O., Schwab, S., Bernard, S., Possnert, G. and Frisén, J.
The age and genomic integrity of neurons after cortical stroke in humans
Accepted for publication, Nature Neuroscience (2014)

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LIST OF SELECTED ABBREVIATIONS

A-EJ	Alternative end joining
AID	Activation induced cytidine deaminase
ATM	Ataxia telangiectasia mutated
BLM	Bloom
BRCA1,2	Breast cancer antigen 1,2
CARs	Cohesin associated regions
CdLS	Cornelia de Lange syndrome
ChIP	Chromatin immunoprecipitation
CSR	Class switch recombination
CTCF	CCCTC-binding factor required for transcriptional regulation
CtIP	CtBP interacting protein
CHK1/2	Checkpoint kinase 1,2
DDR	DNA damage response
DI-cohesion	Damage-induced cohesion
DNA Lig4	DNA ligase 4
DNA PKcs	DNA dependent protein kinase catalytic subunit
Dnl4	DNA ligase 4
DSB	DNA double strand break
Eco1	Establishment of cohesion 1
ESCO1,2	Establishment of cohesion 1,2
Eso1	sister chromatid cohesion protein/DNA polymerase η
ESR	Environmental stress response
FB	Fibroblast
γ -IR	Gamma irradiation
H2A	Histone 2A
HDAC8	Histone deacetylase 8
Hos1	Hda one similar 1
HR	Homologous recombination
Ig	Immunoglobulin
IR	Ionizing irradiation
Ku	Ku70/Ku80 complex
LCLs	Lymphocytic cell line
Lif1	Ligase interacting factor 1
MDC1	Mediator of DNA damage checkpoint protein 1
Mec1	Mitosis entry checkpoint
Mre11	Meiotic recombination 11 homolog A
NBS1	Nijmegen breakage syndrome 1
Nej1	Nonhomologous end joining defective 1

NHEJ	NonHomologous End Joining
NIPBL	Nipped-B-like
PARP-1	Poly (ADP-ribose) polymerase 1
PCNA	Proliferating cell nuclear antigen
PDS5	Precocious Dissociation of Sisters 5
PIP	PCNA-interacting peptide
Rad	Radiation sensitive
RBS	Roberts syndrome
RNAi	RNA interference
RNF168	Ring Finger containing protein 168
RPA	Replication protein A
SA	Stromal antigen
Sae2	Sumo activating enzyme subunit 2
SCC	Sister chromatid cohesion
Sgs1	Small growth suppressor 1
SMC	Structural Maintenance of chromosomes
ss-DNA	single stranded DNA
Tel1	Telomere maintenance 1
TLS	Translesion DNA synthesis
XLF	XRCC4-like factor
UBZ	Ubiquitin-binding-zinc-domain
UV	Ultraviolet
V(D)J	Variable (diversity) joining
WAPL	Wings apart like
WRN	Werner
XLF	XRCC4-like factor
XP-V	Xeroderma pigmentosum variant
XRCC4	X-ray repair cross-complementing protein 4
Xrs2	X-ray sensitivity 2
53BP1	p53 binding protein 1

1 INTRODUCTION

Cell division is fundamental for all living organisms. In higher eukaryotes, cell division is not only vital during the embryonic development but also during the entire lifetime for tissue renewal. Preserved genome integrity is crucial for cell division and to achieve this, the genetic information has to be correctly copied and equally distributed between the daughter cells. Since DNA frequently suffers substantial damage, caused by both endogenous and exogenous damaging agents, an important part of genome integrity is efficient and correct repair of damaged DNA. The cohesin complex has been shown to play a crucial role in genome integrity through its importance for both DNA repair and chromosome segregation. In addition, the cohesin network also plays a critical role in gene regulation. This role is illustrated by the group of human developmental disorders collectively known as Cohesinopathies, which are caused by dysfunctional cohesin. The aim with this thesis was to elucidate the function(s) of the cohesin network in genome integrity by using either the model organism budding yeast (*Saccharomyces cerevisiae*) or human cell lines.

1.1 THE COHESIN COMPLEX

The cohesin complex is named for its canonical role in mediating sister chromatid cohesion (SCC). Initially, it was unknown if cohesion between sister chromatids was achieved by topological intertwinings between the chromatids or conducted by the action of proteins. One of the key findings was when Koshland and Hartwell in 1987 showed that intertwinning of the sister chromatids alone was not sufficient for SCC. Instead, they proposed the existence of one or more proteins whose collective function is to hold chromatids together [1]. Ten years later, Koshland's and Nasmyth's groups identified the cohesin complex [2, 3]. Since then, studies have revealed that SCC is a complex molecular process involving numerous proteins, including both accessory proteins and regulatory factors in addition to the cohesin complex (Table 1). In the subsequent sections, which primarily focus on the complex in *S. cerevisiae*, the composition of cohesin and the regulation of cohesion will be presented.

		<i>Saccharomyces cerevisiae</i>	<i>Homo sapiens</i>
Cohesin core complex	SMC proteins	Smc1 Smc3	SMC1A SMC3
	Kleisin subunits	Scc1/Mcd1	RAD21
	Kleisin binding protein	Scc3	SA1/STAG1, SA2/STAG2
Cohesin regulators	Loading complex	Scc2 Scc4	NIPBL MAU2
	Acetyltransferase	Eco1/Ctf7	ESCO1, ESCO2
	Kleisin binding protein	Pds5	PDS5A, PDS5B
	Pds5 binding protein	Rad61/Wpl1	WAPL
	Deacetylase	Hos1	HDAC8

Table1. Components of the mitotic cohesin complex and cohesin associated and regulatory factors.

1.1.1 Structure and composition of the cohesin complex

Together with condensin and the Smc5/6 complex, the cohesin complex forms a family of large multi-subunit complexes whose cores are built from the evolutionarily conserved Structural Maintenance of Chromosome (SMC) proteins. The core structural components of the cohesin complex are Smc1, Smc3, and the non-SMC protein Scc1 (also called Mcd1) [2-4]. Like all SMC proteins, Smc1 and Smc3 are molecules with globular N- and C- terminal domains, separated by two antiparallel coiled-coil arms connected by a central flexible hinge (Figure 1). By folding back on itself at the hinge domain, each SMC molecule brings the C- and N-termini close to each other forming a globular ATPase “head” domain [5-8]

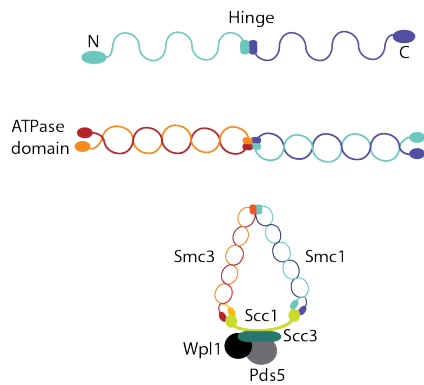


Figure 1. The cohesin complex

(Figure 1). All SMC proteins form dimers. Thus Smc1 and Smc3 form a V-shaped heterodimer, where the kleisin subunit Scc1 bridges the head domains by interacting with Smc1 at its C terminus and Smc3 at its N terminus [6] (Figure 1). The Scc3 protein is the fourth subunit of the cohesin complex, interacting with the complex via Scc1 [6] (Figure 1). In addition to the core components, the accessory proteins Pds5 and Wpl1 have also been shown to interact with cohesin via Scc3 [9-12].

1.1.2 The cohesin cycle

1.1.2.1 The cell cycle

The events that precede the division of one cell into two identical daughter cells together compose the cell cycle known as mitosis. The cell cycle is divided into four phases: two gap phases that separate the two major parts, S phase and M phase. During S phase (S for DNA Synthesis), each chromosome is copied to generate two identical sister chromatids that are compacted and segregated during M phase (M for mitosis). The two gap phases, known as G1 and G2, provide time for growth and allow the cell to monitor the internal and external environment to ensure suitable conditions for S phase and M phase, respectively [13, 14]. During each turn of the cell cycle, the chromosomes must be copied exactly and only once, and then equally distributed between the daughter cells. An important component of this process is the cohesin complex that holds the two sister chromatids together from the time of their synthesis in S phase, until separation at anaphase. Cohesin loading and cohesion establishment are highly regulated, both spatially and temporally, over the course of the cell cycle (Figure 2).

1.1.2.2 Loading of cohesin onto chromatin in G1 phase

In *S. cerevisiae*, cohesin associates with DNA at the end of the G1 phase of the cell cycle, prior to DNA replication [2, 3, 15]. The loading of cohesin to chromosomes requires a protein complex formed by the Scc2 and Scc4 proteins, and the ATPase activity of Smc1 and Smc3 [15-17]. Despite many studies, the knowledge of the molecular mechanism by which Scc2/4 loads cohesin onto chromosomes, and how cohesin then encircles the chromosomes remains poorly understood. Interestingly, it was recently shown that the chromosomal association of Scc2 at the centromere is dependent on cohesin itself. This implies that the assembly of all four subunits of cohesin allows interaction with Scc2/4, and that cohesin and Scc2/4 then associate with chromatin together [18]. Since Scc1 is expressed from late G1, this might explain why cohesin associates with chromatin at this point. The mechanism of how cohesin encircles DNA is not known, but some of the current models will be described in section 1.1.3.2: Holding the sister chromatids together. In mammals, cohesin is loaded onto chromosomes in telophase [19, 20]. The cohesin loading function of Scc2/4 appears to be conserved since all species with identified orthologues of Scc2/4 exhibit cohesion defects if their functions are inhibited [21].

1.1.2.3 Cohesion establishment during S phase

Once cohesin has been loaded, it has the potential to become cohesive. Cohesion is established during S phase, with a strong connection to the replication process. The main regulator of cohesion establishment is the acetyltransferase Eco1 (also called Ctf7) [22-24], which is believed being recruited to chromatin by the proliferating cell nuclear antigen (PCNA) [25, 26]. Eco1 then acetylates the Smc3 subunit of cohesin on two residues: K112 and K113. This acetylation is absolutely required for cohesion establishment, and was shown to inhibit the destabilizing activity of Wpl1 [11, 27-29]. The mammalian genome encodes two Eco1 orthologues, ESCO1 and ESCO2. Acetylation of SMC3 is a conserved step in the regulation of SCC, and the acetylation of the conserved residues K105 and K106 [30] is a reaction that has been shown to depend on both ESCO1 and ESCO2 [31].

1.1.2.4 Maintenance of cohesion during G2 phase

DNA synthesis in S phase and segregation of sister chromatids in mitosis are separated by the G2 phase. Thus, SCC established during S phase must be maintained until anaphase. The acetylation of Smc3 by Eco1 is a key event for maintenance of cohesion, since this modification inhibits the destabilizing activity of Wpl1 and Pds5 [11]. Wpl1 has been shown to be able to destroy cohesion long after replication is complete [32]. Pds5 has been proposed to both promote acetylation and protect the acetylated Smc3. In addition, and somewhat confusingly, Pds5 also has a crucial role in releasing cohesin from chromatin by cooperating with Wpl1 [33]. In mammals, WAPL is antagonized by sororin, which binds to the cohesin complex via PDS5, following acetylation of SMC3 by ESCO1 and ESCO2 [31, 34, 35]. Loss of WAPL function in mammals leads to increased levels of cohesin bound to chromosomes, demonstrating the importance of WAPL in the control of cohesin's association with chromatin, as well as its role in the removal of arm cohesin during prophase [31, 35-37]. In contrast, deletion of *WPL1* in budding yeast results in reduced levels of chromosome-bound cohesin and impaired SCC [11, 38]. This was later suggested to be the result of generally decreased cellular levels of cohesin in *wpl1Δ* cells. It is known that cohesin is also important for chromosome condensation [3, 39]. In line with this, the destabilizing feature of Wpl1 in yeast has been suggested to facilitate the balance of chromosome condensation status [32, 40].

1.1.2.5 Removal of cohesin

To allow for correct segregation of sister chromatids, cohesion must be completely dissolved at anaphase. This is achieved by cleavage of the Scc1

subunit of the cohesin complex by the nuclease separase, leading to opening of the cohesin ring [41]. Securin (also called Pds1), an anaphase inhibitor, prevents the cleavage of Scc1 until anaphase when the anaphase promoting complex (APC), triggers degradation of securin [42, 43]. This releases the securin binding partner separase, which cleaves Scc1, resulting in sister chromatid separation. Once cohesin has been released from chromosomes it is deacetylated by the Hos1 deacetylase. The deacetylation of Smc3 allows both the Smc1 and Smc3 proteins to be recycled in the next cohesion cycle [44-46]. The removal of cohesin from chromosomes is regulated differently in yeast and mammals. In mammals, cohesin is removed in two steps. First, the arm cohesin is removed in a separase independent “prophase pathway”, which depends on WAPL antiestablishment activity and inactivation of sororin [35-37]. Later, at anaphase onset, separase removes the centromeric cohesin as in budding yeast [47]. HDAC8 functions as a mammalian SMC3 deacetylase, which facilitates renewal of cohesin following its removal from chromatin in prophase or anaphase [48].

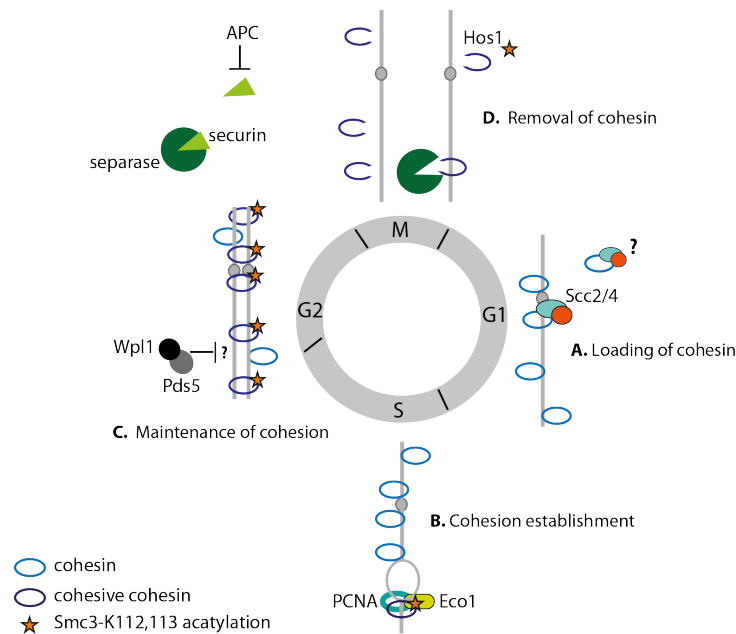


Figure 2. The cohesin cycle in budding yeast. **A.** Cohesin is loaded onto chromosomes in G1 by the Scc2/4 complex. **B.** Cohesion is then established in S-phase, with a strong connection to the replication process. The main regulator of cohesion establishment is Eco1. **C.** The acetylation of Smc3 by Eco1 is a key event for maintenance of cohesion, since this modification inhibits the destabilizing activity of Wpl1 and Pds5. **D.** Cohesin is removed from chromosomes at anaphase through cleavage of the Scc1 subunit by separase.

1.1.3 Cohesin's association with chromatin

1.1.3.1 Localization of cohesin

As mentioned previously, the chromatin association of cohesin depends on the loading complex Scc2/4 [15]. Genome-wide mapping using Chromatin Immunoprecipitation (ChIP) in combination with chip (ChIP-chip) of Scc2 and Scc4 showed an identical localization patterns of these proteins. The binding sites were found next to telomeres, at the centromeres, as well as at numerous places along chromosome arms [49]. In the same study they showed an overall difference in the binding pattern between Scc2/4 and cohesin in *S. cerevisiae*. The most predominant region for common binding of the loading complex and cohesin is at the centromere [49, 50]. This observation led to the assumption that cohesin is loaded at the Scc2/4 binding sites and subsequently translocated away to its permanent positions. Since most of the cohesin association regions (CARs) are found in convergent intergenic regions, with no sequence similarities, it has been proposed that transcription is responsible for positioning cohesin along the chromosome arms [49, 51]. However, it is still not clear if cohesin slides along the chromosomes or translocates to its permanent positions by an alternative mechanism. In addition, Scc2/4 have in one study been mapped to CARs both in pericentromeric and arm regions. These observations are inconsistent with an overall difference in binding pattern between cohesin and Scc2/4, and suggest that cohesin is targeted to CARs largely by the Scc2/4 association at these locations [52].

Cohesin is associated with chromatin throughout the genome at CARs that extend over 1-4 kb, at an interval of 2- to 35-kb [49, 50, 53, 54]. The region on the chromosomes in budding yeast that shows the most notable enrichment in binding of cohesin is the pericentromere, which surrounds the smaller centromere [50, 53, 55-57]. If cohesin is removed from the pericentromere, it leads to increased chromosome loss. This illustrates the functional importance of cohesin localization at these positions [58, 59]. The factors or sequences that attract Scc2/4 to chromatin are not well defined. One factor suggested to be involved in Scc2/4 loading is the kinetochore, which is required for mediating the binding of microtubules to chromosomes [18, 59-61]. Furthermore, Ctf19, one of the members of the kinetochore complex, has been shown to be required for the binding of Scc2/4 to the centromere [18, 60, 61]. Mutations in the same component also result in pericentromeric cohesion defects [60, 61]. The

localization of cohesin in other organisms will be discussed in section 1.5.1: The cohesin network in transcription.

1.1.3.2 Holding the sister chromatids together

The interactions between Smc1, Smc3 and the kleisin subunit Scc1 create a ring-like structure and a model where this monomeric ring encircles the two sister chromatids has been proposed [6]. This model is referred to as the “one ring” or “embrace” model. According to this model, the cohesin ring is opened at the Smc1/Smc3 head domains through hydrolysis of ATP, allowing the chromatids to slide inside. Upon binding to chromatin, a new ATP molecule closes the head domain, and the binding of Scc1 to the heads of both Smc1 and Smc3 stabilizes the ring [6, 62, 63]. In addition, opening of the Smc1/Smc3 hinge has been suggested to be required for DNA entry into the ring [64]. Several additional models, referred to as the “bracelet” and the “snaps” model, have been suggested. Both of these propose oligomerization of the SMC complexes, either at the head (bracelet model), or the hinge domain (snaps model) [65]. In addition, investigation of protein-protein interactions among the cohesin subunits in human cell lines suggests a two-ring handcuff model for the cohesin complex [66].

1.2 DNA DOUBLE STRAND BREAKS

DNA encodes the hereditary information of the cell and it is crucial that the DNA is kept as error-free as possible during cell division. Yet DNA has limited chemical stability and is constantly being exposed to reactive molecules in the cell, resulting in DNA lesions, including single base damages, single strand breaks, and double strand breaks (DSB). For this reason, it was proposed early on that cells must employ efficient DNA repair mechanisms in order to remove such damages [67, 68].

DNA DSBs are considered to be the most toxic type of DNA lesion. The repair of DSBs is fundamental, since this dangerous lesion can lead to large deletions or genome rearrangements and ultimately cell death if left improperly repaired [69]. DSBs can arise during naturally occurring cellular processes such as endogenous oxidative stress or replication fork collapse [70], as well as by external DNA damaging agents like ionizing radiation and different types of

chemicals [71]. DSBs can also be induced purposely to promote genetic diversity. Cellular processes with programmed DSBs include meiosis, the immune repertoire processes of V(D)J recombination and class switch recombination (CSR), and yeast mating-type switching. Regardless of the context of DSB induction all DSBs are repaired via the same basic mechanisms [70, 72].

1.2.1 DNA damage response

Since DNA lesions are a substantial threat to genome integrity, mechanisms have evolved to efficiently monitor and repair DNA damage. Collectively, this system is known as the DNA damage response (DDR) and is outlined in Figure 3. Simplified, the DDR detects the DNA lesions, signals their presence, promotes their repair, and, if required, halts cell cycle progression [69]. The DDR is evolutionarily conserved, and some of the most important steps of the DDR, in both *S. cerevisiae* and mammals, will be discussed in the following sections.

1.2.1.1 DSB recognition, signaling, and checkpoint activation

The first step in the DDR is sensing the damaged DNA. If the damage is a DSB, it is recognized by the MRX complex, composed of Mre11/Rad50/Xrs2 in yeast, and by the MRN complex (MRE11/RAD50/NBS1) in mammalian cells. The Ku heterodimer, consisting of Ku70 and Ku80, is also recruited early to DSB break sites both in yeast and mammals, and seems to be so independently of the MRX or MRN complexes [73-79]. These complexes activate the DNA damage checkpoint by recruiting a protein kinase, Tel1 in yeast [80] and ATM in mammals. Following

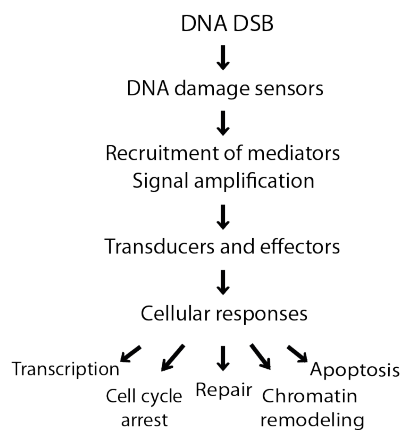


Figure 3. Model for the DDR pathway

recruitment to the break site, Tel1 (ATM) rapidly phosphorylates the histone variant H2A (H2AX in mammals), resulting in an assembly platform for damage response factors [81-86]. In mammalian cells, phosphorylation of H2AX (γ -H2AX) results in recruitment of the mediator MDC1, which has been suggested to generate a positive feedback loop to amplify the γ -H2AX signal by interacting with γ -H2AX, ATM and NBS1 [87]. Subsequent to MDC1, the downstream factors 53BP1 and BRCA1 are recruited to the break site [88, 89]. In yeast, where no

ortholog of MDC1 has been found, the phosphorylation of H2A (γ -H2A) directly facilitates the binding of the 53BP1 ortholog Rad9 [90]. These downstream factors function as molecular adaptors for recruitment of additional proteins to the break site, and are important for phosphorylation of downstream substrates [91].

The presence of Ku and MRX (MRN) at the DSB break site is also important in the choice of appropriate repair pathway and will be discussed further below. The balance between DSB end resection by MRX and end protection by the Ku proteins directly affects the choice of repair pathway [92]. End resection creates single-stranded DNA (ssDNA) tails that are immediately covered by replication protein A (RPA) [93]. An additional protein kinase, Mec1 in yeast and ATR in mammals, is recruited to the DSB break in the presence of RPA-coated ssDNA overhangs [94]. Both Mec1/ATR and Tel1/ATM are key players in the checkpoint response, which coordinate cell cycle progression with DNA repair. The checkpoint activation is a signal transduction cascade where these sensor kinases promote activation of downstream effector kinases, which in turn function to target downstream DDR components as well as amplify the initial DDR signal [95, 96]. In *S. cerevisiae*, Mec1 activates both Chk1 and Rad53 [97] and in humans ATM primarily activates CHK2 while ATR activates CHK1 [98]. If the DNA damage can be managed efficiently and quickly, the lesion can be repaired without induction of cell cycle arrest. However, if that is not the case, the DNA damage checkpoint is activated [99]. Thus, the G1 checkpoint ensures that DNA damage is repaired before the cell enters S phase and the replication process starts [100]. The intra-S phase checkpoint can slow down the replication process if DNA damage occurs [101], and the G2/M checkpoint arrest cells at the metaphase/anaphase transition in response to DNA damage [102].

1.2.1.2 Chromatin remodeling following a DSB

Chromatin remodeling such as post-translational modifications of histones has an important impact on the DDR in mammals and, to a lesser extent, in yeast [103, 104]. Both histone-histone and histone-DNA interactions are altered through phosphorylation, acetylation, ubiquitination, sumoylation, and methylation [72]. As mentioned, phosphorylation of H2A and H2AX is crucial in response to DSBs, since this modification recruits DDR factors plus other chromatin-modifying components, which together are thought to promote DSB repair and amplify DSB signaling [69]. Histone H3 is phosphorylated and histone H4 acetylated in response to DSBs, and are believed to increase the accessibility of the DSB-

flanking chromatin and to stabilize the interaction between MDC1 and H2AX, respectively [72]. In addition, both H2A and H2AX have been shown to be poly-ubiquitinated by the RNF8 ubiquitin ligase in response to DNA DSBs. RNF168 interacts with ubiquitinated H2AX, which amplifies the ubiquitin conjugates, and thereby facilitates the accumulation of factors that act later in repair, such as 53BP1 and BRCA1 near the lesion [105, 106]. Finally, both histone H3 and H4 become methylated during DNA repair, which has been shown to be important for the interaction of 53BP1 [89, 107, 108].

1.2.1.3 DNA damage induced gene expression

The DNA damage-induced transcription is controlled by the DDR signal transduction pathway, and includes sensors, transducers and effectors (Figure 3), but exactly how this is regulated is not fully understood. The majority of the genes affected in response to damage depend on the signaling pathway involving the Mec1, Rad53, Dun1 kinases. However, very few of the genes share a common promoter sequence, suggesting that the downstream effectors are different, but are likely to be transcription factors [109].

A set of DNA repair-related genes are known to be transcriptionally induced in response to DNA-damaging agents, and these can be divided into two classes. The first includes genes encoding proteins that are of importance directly for the repair of damaged DNA, and the second includes genes encoding proteins that primarily function in nucleotide metabolism and DNA synthesis [110-112]. The best-characterized genes in the second class are the *RNR* genes that encode subunits of ribonucleotide reductase. In budding yeast, three out of four *RNR* genes (*RNR2-4*) are repressed by the Crt1 repressor under normal conditions, but in response to DNA damage Crt1 is inactivated by Mec1-Rad53-Dun1 dependent phosphorylation pathway, resulting in expression of the *RNR* genes [113].

Furthermore, in addition to the DNA repair-related genes, several microarray studies have shown that a general stress response pathway, called the environmental stress response (ESR), is transcriptionally induced following break induction. The genes that participate in the ESR fall into two groups: one consisting of genes that are induced, and one that is repressed, following stressful environmental transitions. A majority of the characterized repressed genes are involved in protein synthesis. The reduction of these transcripts, and thereby the synthesis of their products, is believed to help in conserving energy while the cell adapts to the new conditions. The genes whose expression are induced in the ESR

are, in contrast to the repressed genes, involved in a wide variety of cellular processes. The functions of these gene products are believed to be important for the balance of internal osmolarity and oxidation-reduction, as well as for protection of critical aspects such as the integrity of proteins and DNA [114-116].

1.2.2 DSB repair pathways

Two main repair pathways are used to repair DSBs: Homologous Recombination (HR) and NonHomologous End Joining (NHEJ). In addition, an alternative end-joining (A-EJ) pathway has been described more recently and accounts for a fraction of DSB repair. In the subsequent sections, an overview of the three different DSB repair pathways will be presented and the importance of the choice of DSB repair system will be discussed.

1.2.2.1 Homologous Recombination

During HR, an undamaged template is used to restore any sequence information lost at the DSB site. Therefore, HR is considered the most accurate and error-free repair pathway (Figure 4). The initial step during HR is end processing of the DSB, where ssDNA ends are created by 5' to 3' end resection. Recent studies in *S. cerevisiae* have shown that the initiation of HR is a two-step process. First, the MRX complex mediates limited end resection, which is aided by the 5'-3' endonuclease Sae2. These ssDNA ends

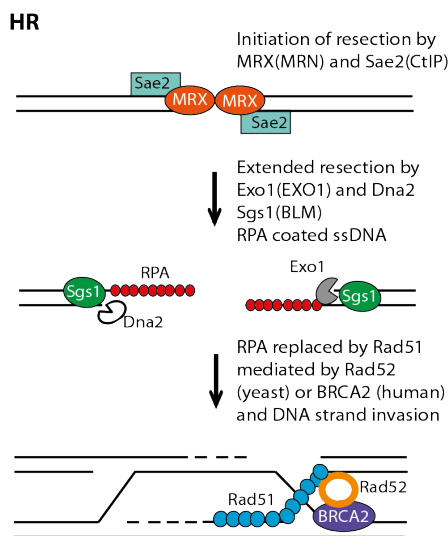


Figure 4. Factors implicated in HR

serves as a substrate for the 5'-3' exonuclease Exo1, and the helicase Sgs1/Dna2 nuclease complex, which performs a more progressive resection [117-120]. A similar two-step model has been proposed in mammals as well [121]. Here, the MRN complex initiates the resection together with CtIP [122], and the following resection is performed by EXO1 and BLM [123]. The ssDNA created during end resection is immediately covered by RPA, thereby preventing ssDNA ends from being degraded. RPA is subsequently replaced by the Rad51 recombinase. This process requires a mediator protein: Rad52 in yeast

and BRCA2 in mammals [124-126]. The next step during HR, which is catalyzed by Rad51, is the invasion of one of the ssDNA ends into a homologous sequence to form a D-loop intermediate. This step is followed by DNA polymerase extension from both 3'-end invading strands. The final steps of HR involve capture and annealing of the second DSB end to the opposing broken strand via the extended D-loop, which leads to the formation of two crossed strands or Holliday junctions (HJs). Resolution of the HJs leads to DSB repair, and results in either crossover or non-crossover products depending on the method of resolution [126,127].

1.2.2.2 NonHomologous End Joining

NHEJ involves direct rejoining of DNA DSBs, and is considered to be the most straightforward repair process (Figure 5). However, if the ends are incompatible for ligation, end processing is required, which results in loss of genetic material. This has led to the concept that NHEJ is error prone [128]. The Ku70/Ku80 heterodimer is thought to be the first set of proteins that bind to the DNA ends during DSB repair through NHEJ [128]. Binding of Ku70/Ku80 has a protective role on the DNA ends, and mediates recruitment of downstream NHEJ factors [129, 130].

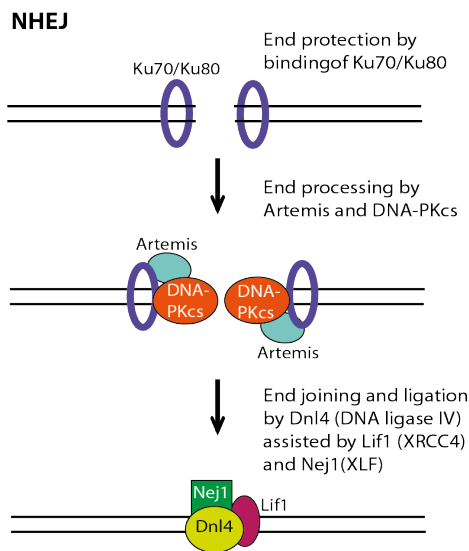


Figure 5. Factors implicated in NHEJ

The Ku proteins are conserved from bacteria to human [131]. In mammals, the Artemis-DNA-PKcs complex interacts with Ku70/Ku80, and has the ability to endonucleolytically cut a variety of damaged DNA overhangs if needed [132, 133]. Though no DNA-PKcs ortholog has been found in yeast, the proteins Tel1 and Mec1 are sequence-related to DNA-PKcs. Therefore, one or both of these proteins could perform DNA-PKcs-related functions in budding yeast [134]. Finally, the ends are ligated by DNA ligase IV together with its co-factors: XRCC4 and XLF in mammals and Dnl4 assisted by Lif1 and Nej1 in yeast [135, 136]; for a review see [137].

1.2.2.3 Alternative End Joining

The A-EJ pathway is the most recently identified DSB repair process (Figure 6). It is still not fully characterized and has been given different names: Backup NHEJ (B-NHEJ) in mammals and Microhomology Mediated End Joining (MMEJ) in yeast. As the latter implies, A-EJ frequently uses microhomologies for DSB repair that might be found at some distance from the break. For this reason, the pathway is considered highly mutagenic [138-140]. A-EJ has been best described during the process of Immunoglobulin Class Switch Recombination (CSR) [141]. Like in HR, the initial step during A-EJ is resection of the DSB ends and involves the MRN complex, as well CtIP [142-145]. The end processing in the A-EJ pathway usually requires less than a 50-nucleotide (nt) resection to expose complementary

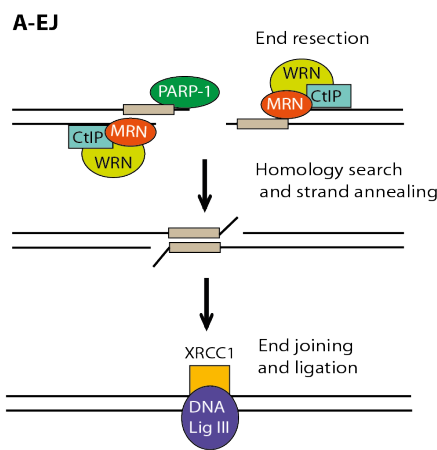


Figure 6. Factors implicated in A-EJ

microhomologies, while HR requires longer ssDNA and thereby utilizes longer homology regions for efficient repair [72]. When a region of sufficient microhomology (2-5nt) has been found, strand annealing of the homologous sequences takes place, creating branched intermediate structures that are trimmed by flap endonucleases [146, 147]. Ligation of the two ends seems to require DNA ligase III [148]. Additional factors implicated in the A-EJ pathway include the DNA end-binding protein PARP-1, the DNA repair protein XRCC1, and the WRN helicase [149-151].

1.2.3 DNA DSB repair - pathway of choice

Though the choice of appropriate DNA repair pathway is vital for cell survival, the method of pathway choice has yet to be fully understood. When a DSB is induced, chromatin modification, proper checkpoint activation, and the early steps of break processing play important roles. On the contrary, programmed DSBs seem to generally be directed into specific repair pathways. For instance, meiotic DSBs are repaired via HR and immune system DSBs are processed by the NHEJ pathway [126]. This could be due to the fact that they are always happening in connection to a certain part of the cell cycle.

NHEJ is active throughout the cell cycle, and is the major DSB repair pathway in G1 phase cells where no close repair template is available. If HR would function during G1, where no sister chromatid is available, it would be forced to use another homologous chromosomal region as a template. This would increase the risk of crossing-over between repetitive sequences, leading to genomic rearrangements, including amplifications, deletions, inversions, translocations, and gene conversions. Thus competition between HR and NHEJ starts in S phase. For example, HR is the preferred DSB pathway following replication fork collapse, where the lesion can be a ssDNA region or a one-ended DSB [72]. In yeast, the cell-cycle-dependent activation of CDK promotes the switch from NHEJ to HR during S/G2 phase [152, 153]. Studies have shown that the levels of several critical HR proteins increase when the cell proceeds from S to G2 phase [154]. However, recent studies in mammalian cells have shown that NHEJ represents the major DSB repair pathway not only on G1 but also in G2 [155, 156].

The initiation of end resection is one of the key steps in the choice between NHEJ and HR in S/G2 phase [121]. In yeast, Ku70/Ku80 has been shown to restrict the access of nucleases to the ssDNA ends, thus inhibiting resection, and thereby HR. Therefore the balance between end resection by MRX and Sae2, and end protection by Ku proteins, directly affects the choice of repair pathway [92]. Both 53BP1 and BRCA1 have been implicated in choice of repair pathway in mammals. The function of respective protein is not fully understood, but 53BP1 has been suggested to mediate NHEJ by inhibiting end resection [157], while BRCA1 has been suggested to promote HR and allow resection by antagonizing 53BP1 [158, 159]. Both HR and A-EJ are initiated by resection of the DSB. End resection factors like CtIP promote A-EJ as well as HR [138, 160]. Ku on the other hand has been shown to inhibit the DSB repair through HR and A-EJ [139, 140, 151, 161]. The balance between CtIP and Ku, and the timing of their recruitment to the DSB, will therefore strongly influence the choice between NHEJ and A-EJ. Since A-EJ is considered an error-prone pathway, the obvious choice is to guide the repair pathway towards HR by ensuring extended resection. In HR-deficient BRCA2 mutants, where the ends are resected, but the formation of Rad51 filaments are disrupted, increased use of A-EJ has been reported [162]. The same shift towards A-EJ has been seen in cells deficient in the NHEJ factors Ku80 and XRCC4 [139, 163, 164].

In addition, there are several examples where chromatin remodeling, following DSB induction, affects the choice of repair pathway. Deacetylation of H3K56 and

H4K16 has been proposed to facilitate NHEJ repair [165], whereas acetylation of H3 has been shown to facilitate ssDNA resection and thereby HR [166]. Moreover, H3K36 dimethylation favors NHEJ by increasing the recruitment of Ku70 and NBS1 [167].

1.3. COHESIN IN DNA DSB RESPONSE

1.3.1 Cohesin and DSB repair

It was early demonstrated that DNA repair efficiency increase tremendously when cells go from the G1 to G2 phase of the cell cycle, suggesting that completion of replication, i.e formation of sister chromatids, is important for DSB repair [168]. Both the cohesin complex and S phase cohesion were later shown to be required for postreplicative DSB repair in budding yeast, presumably by holding the sister chromatids in close proximity and ensuring the presence of an undamaged template that could be used for HR [169]. The DNA repair function of the cohesin complex seems to be conserved. Early studies discovered that the Scc1 ortholog in *S. pombe* (Rad21), rendered cells sensitive to γ -IR [170]. In addition, Scc1-depletion in chicken DT40 cells or RNAi inhibition of Scc1 expression in cells from breast cancer patients results in DNA repair deficiency [171, 172].

1.3.2 DNA damage checkpoints and cohesin

Cohesin has also been implicated in DNA damage checkpoint activation in several studies in human cells. SMC1 was shown to be phosphorylated in an ATM-dependent manner following IR, and to be a component of the DNA damage response network that functions in the S phase checkpoint pathway [173]. Furthermore, expression of an SMC1 protein mutated at these phosphorylation sites (serines 957 and 966) abrogates the IR-induced S phase cell cycle checkpoint [174]. In addition, phosphorylation of SMC3 by ATM has also been reported to play an important role in DNA damage response and to affect the intra-S phase checkpoint [175]. Later it was shown that both SMC1 and SMC3 are phosphorylated as part of the cohesin complex [176]. In the same study, the authors report that the function of cohesin in the G2/M checkpoint is

independent of its ability to mediate cohesion. This was concluded after inactivation of sororin, which is required for cohesion establishment or maintenance but is dispensable for the association of cohesin with chromatin, and for activation of the checkpoint [34, 177]. They furthermore propose that accumulation of cohesin at DNA break sites facilitates the recruitment of checkpoint proteins and the mediator protein 53BP1, which activate the intra-S and G2/M checkpoints [176]. So far, cohesin has not been reported to be directly involved in checkpoint signaling in yeast.

1.3.3 Damage-induced sister chromatid cohesion

Two different fractions of cohesin exist in the cell: one that is stably bound to chromatin after S phase has been completed, and one that continuously associates and de-associates [178, 179]. The later will not become cohesive unless DNA is damaged. This reestablishment of cohesion, in response to DNA damage in G2, is called damage induced (DI)-cohesion.

DI-cohesion differs from S phase cohesion in terms of regulation and factors involved. Similar to S phase cohesion, DI-cohesion depends on the Scc2/4 loading complex, which facilitates recruitment of cohesin also to the DNA breaks [180, 181]. In addition to the Scc2/4 complex, several other factors have been shown to be important for recruitment of cohesin to the break. Among them are the DNA damage response factor Mre11, the kinases Tel1 and Mec1 and phosphorylation of the histone H2A [182]. In response to DNA damage, new cohesion is formed genome wide in addition to loading of cohesin at the break site [183]. Unlike establishment of cohesion during S phase, which depends on replication, DI-cohesion seems to be formed independently of DNA synthesis. This was concluded based on the finding that deletion of Rad52, required for strand invasion during HR, did not affect establishment of cohesion in post replicative cells [182, 183]. The establishment factor Eco1 is absolutely required for establishment of both S phase and DI-cohesion [183]. Importantly, in an undamaged situation, no cohesion should be established after S phase is completed. This is ensured by Eco1 degradation during late S phase that prevents cohesion formation in G2. If DNA damage occurs, Eco1 is stabilized and establishment of cohesion outside S phase is made possible [184]. Genetic evidence suggests that Eco1 acetylates the Scc1 subunit on residues K84 and K210, leading to establishment of DI-cohesion. This acetylation seems to be

triggered by Chk1-dependent phosphorylation of Scc1 at residue S83 [28], and has been proposed to counteract the Wpl1 activity in a similar fashion as the acetylation of Smc3 for S phase cohesion [185, 186].

The formation of DI-cohesion in response to break induction was initially assumed to be required for DSB repair. This conclusion was based on the finding that inactivation of Eco1, which prevents formation of DI-cohesion but does not affect recruitment of cohesion to the break site, renders the cells DNA repair deficient [182]. This may be an oversimplification, however, since accumulating data implies that several factors important for establishment of DI-cohesion in response to DSBs are dispensable for the repair of the induced breaks [187].

1.4 DNA POLYMERASE ETA (η)

During S phase, each chromosome is replicated to generate two identical sister chromatids, and this faithful copying of DNA is essential for inheritance of a complete genome. On top of the exposure of exogenous stress that can lead to DNA damage, the replication process itself generates reactive metabolites that can cause DNA damage. As discussed, it is of great importance for genome integrity that damaged DNA is repaired correctly. Despite that, at times distinct mechanisms are required to temporarily tolerate DNA lesions to contribute to cell survival. The replicative polymerases are blocked at DNA lesions, since they are highly stringent and unable to bypass damaged bases. Blocked replication forks can lead to replication fork collapse, translocations and chromosome aberrations. The translesion synthesis (TLS) DNA polymerases have a more open structure than the replicative DNA polymerases, which make them able to replicate past damaged DNA [188, 189]. In *S. cerevisiae*, three different TLS polymerases are found: Pol η and Rev1, which belong to the Y-family of polymerases [189], and a non Y-family polymerase, Pol ζ (Rev3/Rev7) [190].

Pol η has the unique property of being able to synthesize past DNA lesions induced by ultra violet (UV) light. The gene encoding DNA polymerase η is called *RAD30* in *S. cerevisiae*. Deleting *RAD30* in budding yeast results in hypersensitivity to UV-light and leads to an increase in UV-induced mutation frequency [191, 192]. The function of Pol η is conserved, since humans with the Xeroderma pigmentosum variant (XP-V) disease, have been shown to possess

mutations in Pol η , leading to increased frequency of UV-induced mutations and greatly increased incidence of sunlight-induced skin cancer [193, 194].

During TLS, the replicative polymerase must be displaced and replaced with the most suitable TLS polymerase, a process referred to as the polymerase switch [195]. The key player during this event is PCNA, which becomes monoubiquitinated at Lys164 in response to fork stalling due to DNA lesions. This modification of PCNA directs the replication machinery into the TLS pathway [196]. Budding yeast Pol η contains an ubiquitin-binding zinc domain (UBZ) and a PCNA-interacting peptide (PIP), both known to be required for the interaction with PCNA [197-199]. In addition to its role in TLS, Pol η has been suggested to be involved in HR, by extending the invading strand in a D-loop structure [200, 201].

Interestingly, in *S. pombe* the Eso1 protein is comprised of two domains: two-thirds highly homologous to Pol η , and one-third highly homologous to the *S. cerevisiae* Eco1 [202] (Figure 7). The Eso1 protein is important for both UV damage bypass and for SCC in fission yeast [202, 203]. In addition, for its TLS function Pol η is absolutely dependent on PCNA. Knowing that PCNA is instrumental for recruitment of Eco1 to chromatin and thereby for S phase cohesion, the possible connection between Pol η and establishment of cohesion during S phase and/or in response to DSB induction became very thought-provoking [204].

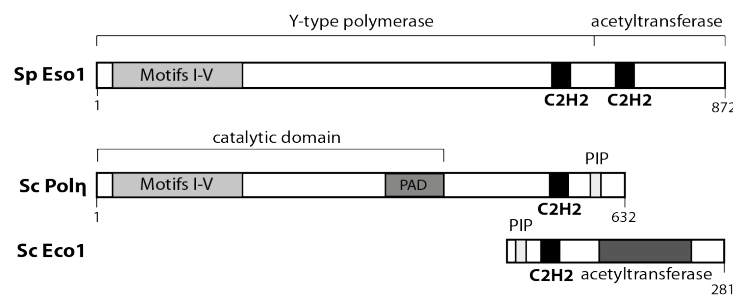


Figure 7. Alignment of fission yeast Eso1 with budding yeast Pol η and Eco1

1.5 FUNCTIONS FOR COHESIN BEYOND COHESION

1.5.1 The cohesin network in transcription

In addition to the canonical function for cohesin in SCC and DNA repair, studies over the last decades have revealed that members of the cohesin network are involved in transcriptional regulation. The first evidence for this came from studies in *Drosophila melanogaster*, where Nipped B (ortholog of yeast Scc2) was found to be involved in the activation of homeobox genes, by promoting long-range enhancer-promoter communications [205]. This is potentially of medical importance since mutations in *NIPBL* (the human Scc2 ortholog), have been found to be the major cause of the development disorder Cornelia de Lange syndrome (CdLS), characterized by upper limb malformations and mental retardation [206, 207]. Both cell lines derived from CdLS patients and cells from a mouse CdLS model (*Nipbl*^{+/-}) display altered transcription profiles compared with control cells [208, 209]. CdLS is one of the diseases collectively termed as “Cohesinopathies”, and will be described further in the following section.

Since the discovery of a transcriptional function for Nipped B in *D. melanogaster*, members of the cohesin network have been shown to be involved in gene regulation in a variety of species. In *Caenorhabditis elegans* and *Xenopus laevis*, MAU2, the ortholog of yeast Scc4, has been implicated in neuronal development [210]. Similarly, inactivation of cohesin in *D. melanogaster* mushroom body γ -neurons, results in axon pruning defects [211]. These results demonstrate a role of the cohesin network in cells not undergoing mitosis, indicating functionally separate roles of cohesin in gene regulation and during the cell cycle.

The regulatory role of cohesin in transcription appears to be conserved across eukaryotes. In *S. cerevisiae*, cohesin was initially suggested to regulate genes by controlling their position within the nucleus [212]. Thus, cohesin was believed to function as a boundary element at the silent loci of HMR, which is involved in Mating type switching in budding yeast [213]. Recent accumulation of data is now expanding the gene regulation function of the cohesin network in budding yeast. Transient inactivation of Scc1 during the G1 phase of the cell cycle caused altered expression of a number of genes with related function in a coordinated fashion [214]. In addition, proteins in the cohesin network were reported to promote ribosomal RNA production [215]. During yeast meiosis, Scc2 has been

shown to regulate gene expression by recruiting cohesin to the chromosomes, where it functions as a transcriptional activator. Furthermore, inactivation of the cohesin subunits Smc1 or Scc3 during meiosis leads to decreased *REC8* mRNA levels as a result of transcriptional inactivation of the *REC8* promoter [216, 217].

The mechanism by which cohesin influences transcription is not well defined. However, the chromatin localization of cohesin and its loading factors in different organisms may give some insight into this. In *S. pombe*, cohesin co-localizes with Mis4 (Scc2 ortholog) at highly expressed genes and localizes between some convergent genes in G2 [218]. Cohesin and Nipped-B co-localize almost completely genome-wide at DNA replication origins, active genes, and at transcription start sites in *D. melanogaster* [219]. In mammalian cells, cohesin has, in addition to the Nipbl binding sites, been shown to bind to same sites as the transcription factor CCCTC-binding factor (CTCF) [220-222]. The chromosomal association of CTCF is required for the subsequent binding of cohesin to these sites, whereas cohesin is dispensable for the CTCFs binding. At the same time, cohesin was shown to be important for CTCF's function as transcriptional insulator [220-222]. Cohesin has also been shown to interact with the Mediator complex and the two complexes co-localize at enhancers and promoters in mouse embryonic stem cells [223]. In addition, cohesin associates with diverse cell type-specific transcription factor binding sites. In breast cancer cells, upon stimulation with estrogen, cohesin co-localizes with the estrogen receptor at its binding sites. Cohesin also associates with liver-specific transcription factor binding sites in human hepatocellular carcinoma cells [224].

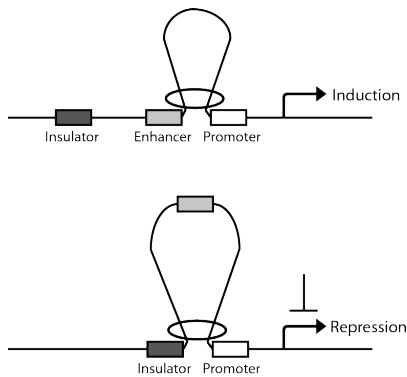


Figure 8. The loop model

Thus, based on the function of cohesin in SCC, where it is believed to encircle the two sister chromatids, the proposed model for how cohesin regulates gene transcription is by forming long-distance DNA loops. These loops generate physical interactions between distant chromosomal loci [205] (Figure 8). Cohesin has been shown to both induce and repress gene expression, and this is believed to depend on whether the enhancer or silencer is brought in close contact with the promoter.

Interestingly, several studies in different model organisms, as well as in human cells, report that reduced levels of cohesin alter gene expression without affecting SCC [205, 209, 225, 226]. In budding yeast, Koshland and co-workers utilized a method to generate systematic reduction of cohesin to investigate if different *in vivo* concentrations of cohesin are required to execute its distinct biological functions. They showed that as little as 13% of the normal cohesin level is sufficient to saturate the preferential CARs in the centromere and pericentric regions and to maintain the SCC function. However, 30% of the normal cohesin level was not enough for binding to the low affinity arm CARs or for accurate condensation or DNA repair [227]. These findings might explain how mutations affecting this complex can specifically lead to cohesin disorder without compromising cell division.

1.5.2 Cohesinopathies

Human diseases caused by defects in cohesin functions are collectively called Cohesinopathies, and comprise the developmental disorders Cornelia de Lange Syndrome (CdLS, OMIM#122470, #300590 and #610759), and Roberts Syndrome (RBS, OMIM#268300)/SC phocomelia (SC, OMIM#269000)). In addition, increasing evidence has revealed a link between impairment of the cohesin network with different forms of human malignancies.

1.5.2.1 Cornelia de Lange Syndrome

CdLS is a genetically heterogeneous dominant developmental disorder. Brachmann first described the syndrome in 1916, but in the 1930s, Cornelia de Lange characterized the diagnostic criteria of the syndrome. Some examples in the literature refer to the disorder as Brachmann-de Lange syndrome; however, it is more widely referred to as Cornelia de Lange syndrome [228]. The syndrome is characterized by craniofacial anomalies, growth retardation, intellectual disability, upper limb defects, hirsutism, and perturbations of heart, kidney, genital, and gastrointestinal development. Clinically, CdLS phenotypes can range from very mildly affected individuals, with no structural abnormalities and minor intellectual disability, to severely affected individuals with upper limb defects and severe intellectual disability [208, 229]. The prevalence of CdLS has been estimated between 1:10,000 and 1:50,000 live births [230, 231]. Approximately 65% of CdLS probands clinically diagnosed have mutations in one of the cohesin-associated genes (*NIPBL*, *SMC1A* or *SMC3*), where the majority of those patients

(60%) possess a heterozygote mutation in the *NIPBL* gene [206, 207, 232-235]. Mutations in the genes encoding the cohesin accessory factors HDAC8, and PDS5 have also been linked to CdLS [48, 236]. Based on the available data the common idea seems to be that the developmental defects seen in CdLS are caused by transcriptional dys-regulation during development, for a review see: [237].

1.5.2.2 Roberts Syndrome and SC phocomelia

Roberts syndrome (RBS) and SC phocomelia are autosomal recessive genetic disorders caused by mutations in the *ESCO2* gene (homolog to yeast *ECO1*) [238, 239]. The clinical features of RBS/SC phocomelia are distinct from CdLS but share some similarities. Patients with RBS tend to have both upper and lower limb defects, mental retardation, and craniofacial defects that include microcephaly, ear malformation, cleft lip and palate, and an undersized jaw [240]. SC phocomelia is a milder form of RBS in terms of physical defects and mental retardation. Most of the mutations found in RBS patients specifically disrupt the acetyltransferase domain of *ESCO2*, indicating that the acetyltransferase activity is essential for the development of the major organ systems defective in RBS [241].

1.5.2.3 Cancer

Increasing evidence has revealed a link between impairment of proteins in the cohesin network with different forms of human cancers. Mutations in *SMC1A*, *NIPBL*, *SMC3* and *STAG3* have all been found in colorectal cancers [242]. In addition, *RAD21* alterations occur in breast cancer, prostate cancers and leukemia [172, 243, 244]. Furthermore, overexpression of *WAPL* has been observed in cervical cancers [245, 246], and in a similar manner elevated levels of *ESCO2* has been implicated in human cancer [247]. However, it is not known whether the aneuploidy and tumorigenesis observed in these tumor cells with impaired cohesin function, are due to altered gene expression or due to chromosomal missegregation, or deficient DNA repair. One of the future challenges is to understand how dysfunctional cohesin contribute to cancer development.

2 COMMENTS ON METHODOLOGY

2.1 EXPERIMENTAL SYSTEMS

Several different experimental systems were used in the papers included in this thesis. Here the most important techniques will be briefly described and benefits as well as challenges will be discussed.

2.1.1 DI-cohesion assays

The function of Polh in DI-cohesion was investigated in paper I. To monitor DI-cohesion, it is important to distinguish between S phase cohesion and DI-cohesion formed in G2 following DNA damage. To do this, three types of experimental systems were used. The first two systems are based on the expression of galactose-inducible wild-type *SMC1* or *SCC1*, in cells where the endogenous *SMC1* or *SCC1* allele is temperature-sensitive (ts) (*smc1ts/Smc1WT* and *scc1ts/Scc1WT*). This allows inactivation of S phase cohesion created by the respective ts-allele by shifting cells to restrictive temperature. Simultaneously, the galactose-inducible WT allele is expressed and used to create DI-cohesion in response to break induction in G2. In these two systems, the cells are kept in G2 throughout the entire experiment. Thus, if the strain tested is DI-cohesion defective, the sister chromatids will spontaneously fall apart following the temperature shift, since the S phase cohesion is destroyed. The third system is based on expression of a noncleavable version of *Scc1* (*scc1^{NC}*). In this situation, the sisters will continue to stay cohesed at anaphase, if DI-cohesion has been induced [180, 182, 183]. For a schematic illustration of these systems see Figure 1 in Paper I.

DSBs were introduced either by use of the HO (HOmothallic switching) endonuclease under control of a galactose-inducible promoter (*pGAL-HO*), or by γ -irradiation (γ -IR). The budding yeast HO endonuclease normally promotes the mating type switching by cleavage of the *MAT* locus on Chr. III. We used either the endogenous break site on Chr. III or HO break sites introduced elsewhere in the genome after deletion of the endogenous HO site.

The different systems for detection of DI-cohesion have their strengths and weaknesses. When using systems based on ts alleles, it is important that the insertion of the ts allele into a strain with mutation or deletion of the gene of interest, does not cause a combined synthetic problem for S phase cohesion. Furthermore, these assays are based on an extended G2 arrest, which should be taken into consideration, since the cells might not behave in a biologically normal manner at the end of the experiment. Determining sister separation is straightforward, however, since there is no ambiguity whether the cell is one G2 cell or two G1 cells. In the *Scc1^{NC}* system, the S phase cohesion is established by WT cohesin, which is an advantage when compared to the ts system. The possible issue with this assay is that it is based on a mutated *Scc1* allele that can easily be reverted to WT or be deleted since the selection for the normal gene copy is strong. Therefore, both its expression and its inability to be cleaved off from the chromosome needs to be carefully monitored. In conclusion, using different approaches to answer the same question has given us confidence that the similar results obtained with the varied methods indeed reflect the *in vivo* situation.

2.1.1.1 Dot system

The DI-cohesion assays are combined with an assay where detection of DI-cohesion is based on the Tet-repressor-GFP/Tet-operator system. In this system, an array of Tet-operators are inserted at the *URA3* locus on ChrV, which then bind to endogenously expressed Tet-repressors with a GFP tag in order to produce a fluorescent signal. This results in one GFP focus in the cells where the sisters are cohesed and two foci where they are separated, which can be visualized in the microscope [180, 182, 183] (Figure 9).

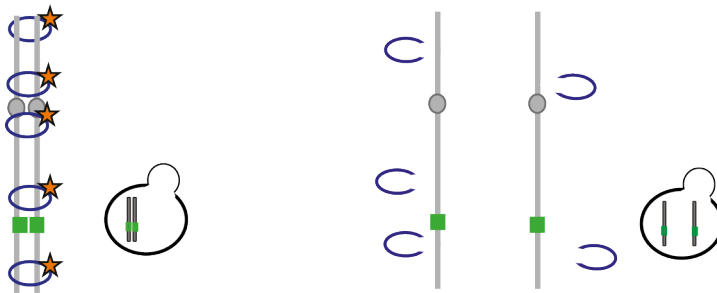


Figure 9. The “Dot system”

2.1.2 Microarray analysis

In paper II, the transcriptional profiles of Scc2-deficient cells, both in the presence and absence of DSB induction, were analyzed using the GeneChip Yeast Genome 2.0 Array (Affymetix). Experimental design is always important, but it is especially critical when performing microarray analysis due to the sensitivity of the assay. Thus, we tried to minimize non-biological variations as much as possible during our microarray study. We used pairs of *S. cerevisiae* strains that were genetically identical in all aspects except for the presence of the recognition site for the HO enzyme. All strains were grown at permissive temperature (21°C) during the G2/M arrest before the temperature was raised to 32°C for 30 minutes, which is restrictive for the ts allele *scc2-4*. Galactose was then added to all strains in order to activate the HO enzyme and induce a single DSB on Chr VI. Total RNA was isolated 90 minutes after galactose addition. Both the quantity and quality of the RNA was assessed before cDNA was synthesized and submitted to the Karolinska Institutet core facility for Bioinformatics and Expression Analysis (www.bea.ki.se), where hybridization to the GeneChip Array was performed.

Ideally, all samples should be collected the same day for the same experiment. In addition, the samples should be hybridized to the same array batch, on the same day, and by the same person. This is often not possible, however, and screening the microarray data to control for technical variations is of great importance. The pre-processing includes background correction, quantile normalization and summarization. Each run was also checked for technical variation and adjustments for batch effects were carried out. A more detailed description of how the analysis was performed is found in Paper II.

2.1.3 ChIP

In paper I and II, chromatin immunoprecipitation followed by microarray (ChIP-chip), sequencing (ChIP-seq) or real time PCR (ChIP-qPCR), was used. ChIP techniques are now standard for mapping of protein-genome interactions. In short, the proteins are cross-linked to DNA *in vivo*, the cells are then lysed and the DNA fragmented by sonication. Thereafter, crosslinks are reversed and the immunoprecipitated DNA is purified. For ChIP-chip, the immunoprecipitated DNA is amplified and hybridized to oligo probes on a microarray [248]. For ChIP-

seq, the DNA fragments of interest are instead sequenced directly [249]. Genomic sequencing following ChIP is today the dominant and most preferred approach for studying protein-genome interactions [249-251].

The different ChIP-techniques appear to be straightforward, but there are technical aspects of the procedure that are difficult to control and could complicate the analysis. Fixation, epitope accessibility, and antibody specificity are a few of the factors that can drastically alter results. In addition, because of the known biases in chromatin preparation and sequencing, it is important to compare the mapped reads of the immunoprecipitated sample to an input control of chromatin that is crosslinked but not immunoprecipitated. Neither ChIP-chip nor ChIP-seq is considered quantitative. Therefore, ChIP-qPCR is often used on selected regions to investigate/confirm differences in binding. Both the benefits as well as the challenges in exploiting these techniques have been carefully described elsewhere [248, 249].

In addition, a recently published study showed that one artifact in ChIP is the reproducible, but biologically non-significant, enrichment of proteins at highly expressed genes. The enrichment in binding at these positions was shown to be caused by high levels of transcription by polymerase II and III [252]. Therefore, the authors suggested that a heterologous protein control should be included in ChIP experiments in order to distinguish between biologically significant enrichments and artifacts.

2.1.4 CSR junctions

In paper III, class switch recombination (CSR) junctions from *in vivo* switched B cells were analyzed. CSR is a physical process that induces DSBs to create genetic rearrangement enabling immunoglobulin (Ig) diversity. B cells undergo Ig class switching *in vivo* after immunization or infection. The process is initiated by the B-cell-specific factor AID (Activation-Induced cytidine Deaminase), through DNA deamination, resulting in DSBs in the donor and acceptor switch (S) regions [253]. These breaks are processed and repaired during G1, by recombination of the two S regions [254], where NHEJ is considered to be the primary mechanism used for the DSB repair (Figure 10). First, genomic DNA is purified from peripheral blood lymphocytes. Analysis of DNA sequences at or around the recombination site is done by use of a nested-PCR approach where the fragments

of recombined S regions are amplified [255]. The PCR products were purified and subsequently cloned into a vector, transformed into competent bacteria, and then sequenced [256]. The switch recombination junctions were determined by aligning the switch fragment sequences with the reference $S\mu$, $S\alpha 1$, or $S\alpha 2$ sequences.

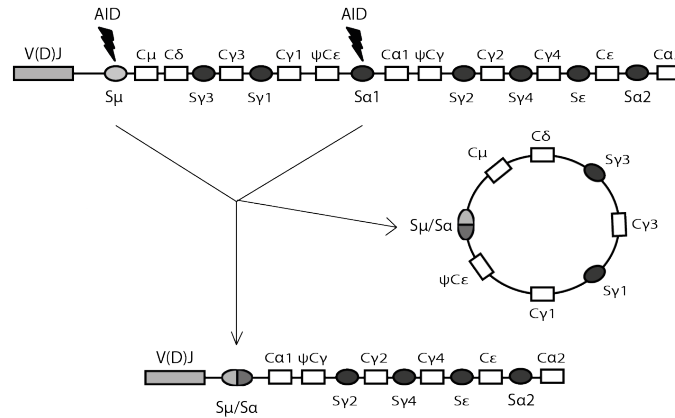


Figure 10. Deletion model of CSR. In this figure, the $C\mu$ gene is replaced by a $C\alpha 1$ gene, resulting in a change from IgM to IgA1 production. The intervening sequence is excised as a circular DNA and the $S\mu$ and $S\alpha 1$ region are recombined, resulting in a $S\mu$ - $S\alpha 1$ recombination junction.

Suggested minimal information required for S-S junction analysis has been well reviewed in [256]. Two examples of what is important during the analysis are firstly, usage of age matched controls, since the average length of microhomology is significantly longer in pediatric controls than in adults [257, 258]. Secondly, analysis of more than one type of CSR junctions is recommended, since it is evident that in human B cells, $S\mu$ - $S\alpha$ and $S\mu$ - $S\gamma$ junctions are resolved differently in WT cells as well as in cells with deficiencies in various DNA repair factors [259-261]. It is also important to keep in mind that this is an assay where the “events” analyzed originate from an immune response that has taken place in each individual during their lifetime. This is both a strength and a drawback. The strength is that it reflects a biologically relevant reaction, but it is obviously not a controlled experiment with many individuals, patients and controls that are exposed to the same antigen during the same conditions. Therefore, the *in vitro* plasmid-based assay was a relevant complement to the CSR junction assay.

2.1.5 Model organisms

2.1.5.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae (budding yeast) is an unicellular eukaryote with many properties that makes it an ideal model organism. Budding yeast has a short generation time and it is relatively easy and cheap to maintain. In addition, it has a highly efficient DNA recombination system that enables *in vivo* recombination of transformed linear DNA with homologous genomic DNA. This makes gene deletions, gene substitutions, epitopic tagging and gene modification relatively straightforward. Budding yeast propagates in both haploid (vegetative) and diploid state (sexual). The haploid genome is made up of 16 chromosomes that are between 200 and 2200 kb in size. The entire genomic sequence was mapped in 1996 and was the first complete eukaryotic genome sequenced. It is also non-pathogenic and requires virtually no precautions for handling. In paper I and II budding yeast was used as model organism.

2.1.5.2 Human cell cultures

In paper III, Epstein Barr Virus (EBV) immortalized B-lymphocytes (LCLs) and fibroblasts (FB) derived from CdLS patients, and unaffected individuals were used. Working with human cell lines has a profound benefit in biology and medicine, compared to animal models, since they are cost effective, owing to features like accelerated growth and minimal nutrient requirements. LCLs are developed by infection of peripheral blood lymphocytes with EBV, giving rise to an actively proliferating B cell population [262]. LCLs exhibit minimum somatic mutation rate in continuous culture [263], LCLs also provide an unlimited source of DNA, RNA or proteins and are a promising *in vitro* model system for genotype-phenotype correlation studies. [264]. As described above, LCLs derived from CdLS patients used in our study, seem to be a suitable tool. However, when studying differences in survival, and proliferation, different transformation efficiency could potentially influence the results. Therefore, in addition to the LCLs, we used primary FBs originating from CdLS patients and control FBs treated with *NIPBL* siRNA, which typically resulted in >70% reduction of the *NIPBL* protein levels. Rewardingly, all three different types of cell lines showed the same DNA repair deficiency compared to control cell lines.

All human studies were performed according to the Declaration of Helsinki and received appropriate ethical approvals.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Importance of Pol η for Damage-Induced Cohesion Reveals Differential Regulation of Cohesion Establishment at the Break Site and Genome-Wide

This study was initiated based on the knowledge that the Eco1 homolog in *S. pombe*, Eso1, is expressed as a fusion protein comprised of two domains: one highly homologous to Pol η , and one highly homologous to Eco1 [202]. Since Eco1 is absolutely required for both S phase and DI-cohesion [22-24, 183], we aimed at investigating the importance of Pol η for DI-cohesion in *S. cerevisiae*. In doing so, we used three different DI-cohesion assays (described in the section of experimental systems), and found that Pol η indeed is required for formation of DI-cohesion in G2, both in response to γ -IR and induction of a single DSB. DI-cohesion has been proposed to be important for efficient repair of DSBs in G2, since inactivation of Eco1 in G2, which prevents the establishment of but DI-cohesion, but not loading of cohesin to the break, rendered the cells DNA repair deficient [182, 183]. Therefore, we continued to investigate the postreplicative DSB repair capacity in cells lacking Pol η . To our surprise, despite its importance for formation of DI-cohesion, Pol η was not required for the DSB repair.

It is known that cohesin in response to a DSB, is loaded around the break and that DI-cohesion is formed genome wide and presumably in the DSB region [180, 181]. The importance of the cohesin complex at the break site can be explained by its cohesive function, holding the sister chromatids in close proximity, and thereby enabling the HR mediated repair. Since Pol η was shown to be required for cohesion formation, but not for loading of cohesin at the break site or the subsequent repair, we decided to test if there was a difference in how the cohesion was formed at the break site and genome wide. To address this, we used a system that allowed us to study the formation of cohesion close to the break site exclusively. We could indeed confirm our hypothesis that genome-wide and DSB proximal DI-cohesion seem to be regulated differently. Since Pol η was shown to be dispensable for break proximal cohesion, for loading of cohesin to the break site and for the subsequent repair, we speculated that the break

proximal cohesion is important for the repair. To strengthen this hypothesis, it would be interesting to test additional proteins known to be involved in formation of DI-cohesion but not essential for DSB repair and vice versa, using the same system. *chk1Δ* cells show the same phenotype as Polη-deficient cells in that they too lack genome-wide cohesion despite normal cohesin loading at the break and repair following damage [183, 187], making this gene a suitable candidate. According to our “model,” *chk1Δ* cells would also be dispensable for break proximal cohesion. A positive control for a protein required for DSB proximal cohesion could be Rad52, which has been shown to be important for DSB repair. Even though the loading of cohesin to the break has not been investigated in a *rad52Δ* strain, it has been shown to be dispensable for genome wide DI-cohesion[182, 183].

Polη has a well-defined function during TLS, where it is regulated by PCNA [197-199]. Therefore, we created mutants in which the interaction with PCNA was abolished. We also made TLS polymerase-dead mutants of Polη and investigated the ability of all these to form DI-cohesion. Inhibition of PCNA interaction had no effect on the formation of DI-cohesion; neither did polymerase dead mutants, with the exception of one (*rad30-D155A*). This suggested to us that the function of Polη in DI-cohesion is different from its function in TLS. We do not have any obvious explanation for why the Polη -*D155A* mutant was DI-cohesion-. It might be that the D155 amino acid is crucial for chromatin association of Polη, since it is responsible for liganding of the two essential Mg²⁺ ions in the active site of the polymerase.

It has previously been reported that overexpression of Eco1 in G2 bypasses the requirement for a DSB to induce DI-cohesion [183]. This, in combination with the knowledge about the Eco1 protein in *S. pombe* [202], led us to investigate whether excess amounts of Eco1 could rescue the DI genome-wide cohesion defect in the absence of Polη. We found that both overexpression of *ECO1* and an acetyl-mimic version of one of the Eco1 acetylation targets, Scc1 (*scc1-K84Q,K210*), could rescue the defect in DI genome-wide cohesion observed in Polη-deficient cells. This suggested that Polη is important for the function of Eco1. Since we were unable to show a direct interaction between Polη and Eco1, an alternative explanation could be that Polη and Eco1 conduct their functions in parallel pathways and that overexpression of the Eco1-dependent pathway compensates for the loss of the other. Alternatively, the interaction may be transient and not possible to detect with IP techniques. The relationship between

Pol η and Eco1 in *S. cerevisiae* has to be further investigated in order to decipher the mechanism behind their activities.

By examining the function of Pol η in DI-cohesion, we were able to get a better understanding on how this process is regulated. Even though the mechanism of how Pol η conducts its function still remains unknown, it seems likely that DI-cohesion is regulated differently at the break site and genome-wide. In addition to being differentially regulated, the function of break-proximal and genome-wide DI-cohesion might also be different. We suggest that genome-wide DI-cohesion is important for correct chromosome segregation. To investigate the consequence of deficient genome-wide DI-cohesion, we re-exposed the cells to multiple rounds of DSBs and found that after the fourth repetition of damage induction, the survival rate of Pol η -deficient cells compared to WT cells had reduced significantly. This suggests that the DI genome-wide cohesion has an important function for maintenance of genome integrity in the presence of DNA damage.

3.2 Paper II

Inactivation of the Cohesin Loader Scc2/4 alters Gene Expression both Globally and in Response to a Single DNA Double Strand Break

In this manuscript we aimed at examining the transcription profiles in WT and Scc2-deficient budding yeast cells in the absence and presence of DNA damage. This was done since, in addition to their role in SCC, cohesin and Scc2/4 are essential for correct DNA repair and have been shown to be involved in gene regulation in a variety of species. By surveying the transcriptional profiles, we found that 473 genes were differentially expressed in Scc2-deficient cells compared to WT cells in the absence and 632 genes in the presence of break, among 5841 open reading frames (transcripts) examined. Among the differentially expressed probe sets, 168 probe sets were uniquely affected in the absence of break and 355 in the presence of break in Scc2-deficient cells compared to WT.

These microarray datasets were analyzed according to biological process, using Saccaromyces Genome Data base Gene Ontology (SGD GO) slim mapping [265]. The genes were divided into up- and down-regulated genes and processes that

showed a significant enhancement compared with genome frequency ($\text{FDR} \leq 0.05$) were considered further. This analysis showed that most of the genes with altered expression in *scc2-4* cells compared to WT cells, were involved in processes that were affected independently of break induction. However, several things suggested that also the transcriptional response to DNA damage was abrogated in the absence of functional Scc2. First, a larger number of genes was affected the presence of break, comparing WT and *scc2-4* cells. Second, in WT cells, we found the processes “DNA damage”, “DNA repair” and “DNA recombination” to be enhanced in the presence of DSBs, which was not the case in *scc2-4* cells. Lastly, in WT cells several of the up-regulated DNA damage response genes were found in the group of probe sets exclusively affected in the presence of DSB.

Since the first experiment indicated that Scc2 would be important also for the transcriptional response induced by DSB formation, we started to investigate if a single DSB would induce a response analogous to what had been reported for IR, UV, MMS, HU, and 4-nitroquinone [115, 266, 267]. In WT cells a total of 113 genes displayed statistically significant difference in expression between absence and presence of one DSB. This response was severely altered after inactivation of Scc2, where 976 genes were transcriptinally affected after break induction – almost a ten-fold increase. Although a majority of the previously known DNA damage induced genes were induced in both WT and *scc2-4* cells in response to DSB, a difference between WT and *scc2-4* cells could be seen for genes encoding proteins in the cohesin network. Using quantitative real time PCR (qRT-PCR), we could confirm the results achieved with microarray for a set of DNA repair and cohesin network genes.

These two data sets were further analyzed using SGD GO slim mapping as for the first experiment. The two most significantly enhanced processes in the group of up-regulated genes in WT cells were “cellular response to DNA damage stimulus” and “DNA repair”. None of these were enhanced in Scc2-deficient cells; instead, completely different processes such as responses to chemical stimuli, oxidative stress and starvation were significantly enhanced. This confirms the result seen in the initial experiment that, in addition to the general transcriptional defect in G2, absence of Scc2 also affects the transcriptional program induced in response to DSB induction. Studying the repressed processes in Scc2-deficient cells strongly indicated that, in the absence of Scc2, processes involved in ribosome production and function were impaired. Interestingly, Smc1 and Eco1, proteins in

the cohesin network, have been implicated in promoting ribosomal RNA production and protein translation [215]. The importance for the ribosomal processes could therefore be common for all the proteins in the cohesin network.

The canonical function of Scc2 is to load the cohesin complex onto chromosomes [15-17]. Therefore, we wanted to investigate if the transcriptinal dysregulation detected in the absence of Scc2 could be an effect of defective cohesin loading in G2/M. However, no apparent differences in the genome wide cohesin binding was seen between WT and Scc2-deficient cells using chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) of Scc1. Therefore, we concluded that the different transcription profile detected in Scc2-deficient cells cannot be attributed to removal or formation of new cohesin binding regions at new positions in the genome. One must also take into account that the Scc1 binding detected, results from both G1-loaded cohesin and cohesin loaded in G2 after damage. It would be interesting to investigate specifically the G2-loaded cohesin. This can be done using an affinity tagged version of Scc1 expressed solely in G2.

A difference in loading of cohesin between WT and Scc2-deficient cells was seen, as previously reported, at the break site where less cohesin was bound in the absence of Scc2. In line with this, the majority of genes surrounding the break showed reduced expression after break induction in WT cells. On the other hand, Scc2-deficient cells transcriptionally repressed only half (3/6) of the most DSB-proximal genes on the microarray. This result was confirmed using qRT-PCR. Interestingly, it has previously been reported that transcription of genes in close vicinity of the natural HO-cleavage site are repressed in response to break induction [268]. Our results indicate that in the absence of Scc2, less cohesin is recruited to DSB region, which might influence the transcription of the break-proximal genes. One explanation could be that cohesin, by binding to the area, specifically prevents or actively silences the expression of these genes in response to break induction. Further investigations must be done in order to prove this idea, but it is an interesting possibility to keep in mind.

From this study, we conclude that transient inactivation of Scc2 in G2 has effects on general transcription, both globally and in the DSB-proximal region. Whether this is a result of a function mediated by cohesin, or by Scc2 itself, is still not clear. The fact that we do not see any difference in cohesin binding in G2 in the absence of functional Scc2 argues for a cohesin-independent role for Scc2. As discussed,

however, the G2-specific binding of cohesin in response to break induction in the absence of Scc2 has to be investigated to solidly confirm this. In addition, it would be interesting to examine the transcriptional response in the absence of Scc4, the binding partner of Scc2, to answer the question of whether Scc2 has a function independent of Scc4. Intriguingly, a recently published study showed that NIPBL (human ortholog of Scc2) localize to different chromosomal regions than cohesin. NIPBL knockdown was in addition shown to reduce transcription differently than cohesin knockdown. Finally, they showed that NIPBL but not cohesin, was binding to the promoter regions of these active genes [269].

3.3 PAPER III

A regulatory role for the cohesin loader NIPBL in nonhomologous end joining during immunoglobulin class switch recombination

The aim with this paper was to examine the involvement of NIPBL, the human homologue of Scc2, in DNA DSB repair. B-lymphocytes (LCLs) and primary fibroblasts (FB) derived from patients diagnosed with CdLS were used. In addition, cells from healthy individuals, ATM or Cernunnos-deficient patients, and RBS patients, were used as controls.

Initially the DNA damage sensitivity was investigated using a MTS proliferation assay that determines the relative number of viable cells following exposure of increased dosages of γ -IR. Low doses of γ -IR caused a significantly reduced survival in all CdLS LCLs compared to the control cells. This radio-sensitivity was confirmed in FBs from CdLS patients, and control FBs treated with *NIPBL* siRNA, using a colony formation assay. Since LCLs are EBV transformed, the differences in survival and proliferation could be affected by different transformation efficiency. Thus, the colony formation assay using the FBs from CdLS patients, and control FB treated with *NIPBL* siRNA, was a valuable experiment. One puzzling observation at this point was that the survival of the patient cell lines was decreased despite the fact that the DNA repair capacity measured by the comet assay was the same between patients and controls (data not shown). In addition, when determining the cell cycle profiles of the LCLs it was shown that the majority of the cells (62-88%) were in the G1 phase of cell cycle. Since NHEJ

is the predominant DNA repair pathway in G1, these results raised the possibility that NIPBL may be important for NHEJ in addition to HR, as previously described. To further understand how DSB are repaired in CdLS cells, we used three different experimental systems. First, CSR junctions from *in vivo* switched B cells were analyzed (described in the section of experimental systems). The result showed a significantly enhanced proportion of junctions with microhomologies (MH). Furthermore, a reduced proportion of $\Sigma\mu$ - $\Sigma\alpha$ junctions with direct end joining were found in NIPBL-deficient B cells (i.e. no sequence MH), compared to healthy controls. The number of junctions with 1 bp insertions was also significantly reduced. This pattern largely resembles switch regions analyzed in cells with known dysfunctional NHEJ pathway where a backup pathway depending on MHs is frequently used, suggesting the involvement of NIPBL in the NHEJ process.

An *in vitro* plasmid based assay was used in the second system. Here, a linearized plasmid with defined blunt ends was transiently transfected into FBs from CdLS patients, a FB cell line treated with NIPBL siRNA, or control FBs. The contribution of direct joining was estimated by sequencing [270]. The proportion of direct joining was significantly reduced in both NIPBL-deficient cells and the NIPBL knockdown cells compared to the control. At the same time, the proportion of 6-bp MH-mediated end joining was significantly increased.

Lastly, the importance of Scc2/NIPBL for NHEJ in budding yeast was investigated. Since Scc2 is an essential gene, the same temperature-sensitive allele of *SCC2* (*scc2-4*) as in paper II was used. After arrest in G1, a single DSB at the *MAT* locus on Chr. III, was induced by expression of *pGAL-HO*. Since the intrachromosomal regions normally used for its repair were deleted and the breaks was induced on both sister chromatids, these breaks could only be repaired by NHEJ. The results showed that Scc2 might be as important as Lig4 for NHEJ in budding yeast. Compared to *lig4Δ*, a slightly more severe defect was observed in *scc2-4* cells alone and in combination with *lig4Δ*, which could be due to the fact that Scc2 is also required for HR, whereas DNA ligase IV only has a function in NHEJ. When sequencing over the break site, it became apparent that in the surviving WT cells, the HO-induced DSBs were indeed repaired by NHEJ, with most of the recovered junctions having small deletions and insertions, as previously described [271]. Yet most of the amplified sequences derived from the few colonies of surviving *scc2-4* or *lig4Δ* cells were germline at the *MAT*-locus, suggesting that these

surviving cells were probably those few cells that had no DSB induced at this locus.

We demonstrated that NIPBL is important for DSB repair via the classical NHEJ pathway in this work. At this point, no cohesin-independent role of NIPBL had been described, implicating that the involvement of NIPBL in NHEJ may be through the cohesin complex. Moreover, knockdown of the cohesin subunit Rad21/*SCC1* has been shown to result in defects in recruitment of the DNA damage response factor and mediator protein 53BP1 to DSBs [176]. As CdLS patients with deficient NIPBL function have reduced levels of chromatin-bound cohesin [157], we speculated that 53BP1 recruitment might be impaired in the patients too, thus resulting in an increased rate of resection of DSB DNA ends and higher degree of DSB repair via MH-mediated A-EJ. To test this we analyzed the 53BP1 foci formation following γ -IR in NIPBL-deficient and control LCLs. There was a significant difference in the number of foci formed 30 min after γ -IR in the NIPBL-deficient LCLs compared to control cells. This suggests that NPBL is important for proper recruitment of DNA damage response factors such as 53BP1 to DSBs in order to steer the repair towards the NHEJ pathway. This could potentially explain the DNA damage sensitivity observed in cells from CdLS patients, since in the absence of functional NIPBL the cells would be direct towards usage of the more error-prone A-EJ pathway, leading to large deletions and translocations incompatible with survival. In fact, chromosomal rearrangements have been reported in individuals with CdLS [272]. This also further explains the results from the comet assay, where the repair appears to be functional, despite that the cells have difficulties surviving after exposure to γ -IR.

4 PERSPECTIVES AND CONCLUDING REMARKS

Though the canonical role of cohesin is to mediate SCC, evidence is mounting that implicates it in a multitude of other processes as well. Research on cohesin during the last decade has revealed that the cohesin network is involved in many other aspects of cellular processes including DNA repair, transcriptional regulation, and chromosome condensation, to name a few. Thus, the cohesin complex is a master regulator of many processes important for genome integrity. In this thesis, maintenance of genome integrity through activities of the cohesin network has been explored.

In paper I, we studied Pol η and showed that DI-cohesion seems to be differentially regulated at the break site and genome-wide. We also suggest that the functions of break proximal- and genome wide DI-cohesion may be different. We propose that break proximal DI-cohesion is required for repair, while the genome-wide DI-cohesion is important for correct chromosomal segregation. We also provide results suggesting that genome-wide DI-cohesion is important for cell survival following repeated DSB induction. Together, these results imply that the genome-wide cohesion formed after break induction has a significant function for maintenance of genomic integrity in the presence of DNA damage. In addition, our results indicate that S phase cohesion is not sufficient for correct chromosome segregation in the presence of DNA damage. This observation is in line with a recently published report with similar findings [273].

Our results also demonstrate a novel function for Pol η that is not shared with the other TLS polymerases in budding yeast. Then what is the function of Pol η in genome-wide DI-cohesion? As discussed, the connection to Eco1 must be investigated further in order to answer this question. In an attempt to do just this, we have begun to study the post-translational modifications of Pol η following break induction in G2. Since Eco1 is an acetyltransferase and known to acetylate subunits of the cohesin complex, Pol η is a likely target for Eco1 during the process of DI-cohesion. In the future, it would be interesting to investigate if DI-cohesion is a conserved mechanism in higher eukaryotes. XPV patients, whom harbor homozygous mutations in the Pol η protein, suffer a severely increased risk for UV-induced skin cancer. The possibility that they may also have DI-

cohesion deficiencies offers a new field of investigation that may prove useful in bettering the prognosis for these patients.

A gene regulatory role for cohesin and its loading complex Scc2/4 has been described in several organisms but not investigated thoroughly in yeast. In addition, knowledge behind Scc2/4's method of action during gene regulation has been limited. In Paper II, we investigate whether Scc2 is involved in regulation of gene expression in budding yeast. Our results indicate that inactivation of Scc2 in G2 affects the transcriptional response both in the presence and absence of a single DSB. The transcriptional dys-regulation seen by the mere absence of Scc2 compared to WT cells in our study is indeed very similar to what has been reported in studies of human and mouse, which are heterozygous for mutations in their Scc2 homologs. Both these studies report that a large number of genes are affected in the absence of the cohesin loader but with limited fold changes [208, 209].

At this point it is not fully understood whether the gene regulatory defects seen in response to dysfunctional Scc2 is an effect of cohesin being differentially regulated in the absence of Scc2, or if Scc2 itself has a role in transcriptional regulation independently of cohesin. The mechanism by which the cohesin network has been suggested to perform its gene regulatory function in higher eukaryotes is through long-range promoter enhancer interactions [212]. Since it has been shown that a network of inter- and intra-chromosomal interactions make up the yeast genome [274, 275], budding yeast might be a suitable model for studying the gene regulatory mechanisms of the cohesin network. In addition, the human homologue NIPBL has been shown to interact with HDAC and HP1 [276, 277]. It would be interesting, therefore, to investigate if Scc2 is responsible for correct transcriptional programming through shaping of the chromatin landscape.

Until recently, cohesin has only been shown to be important for HR-based DSB repair. By being recruited the site of the DSB, the cohesin complex is believed to tether the sister chromatids together and thereby enforce physical proximity of the template for repair [169]. In paper III, we examine the DNA repair capacity of cell lines derived from CdLS patients and found that NIPBL, in addition to being important for HR, seems to have an important function in the NHEJ pathway. This was concluded after the observation that the patient cell lines displayed an increased usage of microhomology-based, alternative end joining mechanism

during CSR. Our study also suggests that this is a conserved function, since Scc2 was shown to be as important as Lig4 for NHEJ in budding yeast. In addition, our results indicate that the early recruitment of 53BP1 to the break sites is affected in the absence of NIPBL. This recruitment of 53BP1 may work indirectly via cohesin. Absence of 53BP1 has previously been shown to result in increased resection around the DSB in Ig S regions [157], and the participation of cohesin in the recruitment of 53BP1 to DSBs has previously been suggested [176]. If NIPBL/cohesin influence the recruitment of 53BP1 to the site of damage, it might have an important function in the choice of appropriate DNA repair pathway. In fact, Smc1 in budding yeast has been implicated in coordination of DSBs [278]. In line with our results, dysfunctional regulation of repair pathway choice and increased usage of A-EJ is often correlated with increased sensitivity to ionizing radiation [141]. Studying the importance of pathway choice during DNA damage repair and end resection may be of medical relevance. For instance, the increased number of chromosomal rearrangements observed in CdLS cells could potentially reflect a DSB repair deficiency caused by unbalanced end resection [272].

When examining CSR junctions from *in vivo*-switched B cells derived from CdLS patients with NIPBL deficiency, we detected a significant reduction in the proportion of S μ -S α junctions with direct end joining (i.e. no sequence microhomology). This largely resembles switch regions analyzed in cells known to have a dysfunctional NHEJ pathway. Despite that, descriptions of immunological phenotypes of CdLS patients are hard to come by in the literature. No overt clinical manifestations of immunodeficiency were observed in any of the CdLS patients included in the study. Interestingly, a recent study identified for the first time a high frequency of antibody deficiency in CdLS subjects, and they suggested need for screening and management of immunodeficiency in CdLS patients [279]. Studies like this are of great importance since an overall greater knowledge about the CdLS syndrome can lead to better therapies available for these patients.

Taken together, a number of genes in the cohesin network are mutated or mis-regulated in many cancers or developmental diseases. Thus, studies of the cohesin network are medically relevant and are an important area of investigation for the future. The cohesin network is involved in many processes important for genome integrity including SCC, DNA repair, transcriptional regulation, chromosome condensation etc. The multitude of studies that have been, and are currently focused on the cohesin complex and its accessory

proteins and regulatory factors, have increased our knowledge exponentially in recent decade. Though much is known, it is evident that there is much more to learn about this interesting family of protein complexes and how they influence cellular processes critical to genome integrity and survival.

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